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TITLE: Analysis of the Contribution of Stem Cells to Breast Cancer Using Microchimerism-Based Y-Chromosome Stains and Histopathology

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14. ABSTRACT Approximately 15% of mothers to male babies carry in the tissues, Y-chromosome born in cells, believed to come from their baby's stem cell pool. This phenomena was used for tracking incorporation of stem cells from the circulation into breast cancers. The study is not finalized at this stage.					
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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
References.....	None
Appendices.....	7-27

Introduction: Most cells in our body have limited life span. Evidence that supports the existence of a tumor stem cell for breast cancer has given fresh impetus to the search for an adult mammary epithelial stem cell in the normal breast. Such stem cells, licensed for extended tissue repopulation, are proposed to be necessary for cancer tissue maintenance. Compelling data argues, that the cells neighboring the cancer cell, collectively called stroma, are partially responsible for the cancer phenotype and etiology. Therefore, the malignant epithelial cell may recruit stem cells to specific stromal cell populations, thereby providing vital milieu for the cancer development. Embryonic stem cells augment the mother's stem cell pool. Transplanted or chimeric Y chromosome-bearing stem cells behave as do the mothers own: proliferating, differentiating and incorporating into adult epithelial tissue. We view this phenomenon termed Microchimerism, as an opportunity to address stem cell incorporation in cancer tissue. We will access blood samples from 995 breast cancer patients, who had male babies, screen for Y-chromosome DNA and focus on positive patients. Assuming 10% of the patients are micro-chimeric, we would have more than ninety positive patients. Looking at sections of the corresponding cancers we will ask which cells in the tumor (malignant or stroma), if any, have originated from the body's circulating stem cell pool, using the Y-chromosome in micro-chimeric mothers. Such a cell may present new opportunities for the development of novel therapeutic and prophylactic strategies for treating breast cancer.

BRCA1 cancers have been proposed to arise from a stem cell deregulation[1]. Microchimerism offers a unique opportunity to assess that proposal.

Evidence that supports the existence of a tumours stem cell for breast cancer has given fresh impetus to the search for an adult mammary epithelial stem cell in the normal breast[2]. Such a cell might be responsible for routine tissue renewal and the massive expansion in epithelial tissue that the breast undergoes during pregnancy, and might be the cell of origin of most, if not all, breast tumours. Alternatively, the malignant epithelial cell may recruit stem cells to specific stromal cell populations, thereby providing vital milieu for the cancer development. This grant proposal offers a novel method to identify, which cells in the tumours (malignant or stroma), if any, have originated from the body's stem cell pool. Such a cell may present new opportunities for the development of novel therapeutic and prophylactic strategies for treating breast cancer. Embryonic stem cells augment the mother's stem cell pool[3]. Transplanted or chimeric Y chromosome-bearing stem cells behave as do the mothers own: proliferating, differentiating and incorporating into adult epithelial tissue[4, 5]. In our proposal, the Y-bearing cells are assumed to be no more than a marker for stem cell-derived somatic cells: whether they have in any way differentiated; into what cell line; is there any evidence of bias in relation to the type of cells they generate? We argue that any positive results produced by our investigations must be viewed in the context of the patient's overall stem cell activity.

Final Report according to the Statement of Work

IDENTIFICATION OF STEM CELL INCORPORATION INTO BREAST CANCER SPECIMEN USING PATIENTS WITH Y-CHROMOSOME BEARING STEM CELLS DUE TO MICROCHIMERISM.

Task 1. identify the kConFab patients who have microchimerism:

1.

1. To use RT-PCR for Y-chromosome markers on peripheral blood leukocyte DNA (Months 1-3).
Performed by KA

This study has been denied access from the specimen cohort it was designed for (see attached communications). As a result, we had to resort to 200 samples of sporadic breast cancer cases, which we have in Peter MacCallum tissue bank. In the blood of those samples we failed to identify any Y-chromosome DNA, based on a PCR method (using Applied Biosystems TaqMan® technology). The assay was validated to be quantitative for Y-chromosome, using serial dilutions of male blood DNA into a female blood DNA. A major disadvantage of the tissue bank cases is the lack of any record of maternity and gender of child. We are committed to satisfy the kCONFAB committee prerequisites, and go back to the original proposed study. We hope that performance of an animal model experiment, which is aimed to demonstrate stem cell incorporations into the tumor microenvironment, and a reproduction of this experiment in BrCa1 knock out background, will serve as evidence for link to familial cancer, as requested by the kCONFAB committee (see attached novel application for the animal model description).

2. To stain tissue sections of patients found positive in 1. Double Fluorescent stains will utilize one dye for In Situ Hybridization against Y-chromosome markers, and the second dye for immunohistochemistry with antibodies for cell lineage markers (Months 3-12).
Performed by KA

Y-chromosome FISH are working and await identification of the proper maternal breast cancer tissue to stain with.

3. To analyze those stains together with matching serial sections stained with H&E, in order to identify which cell types in the cancer mass are specifically containing these Y-chromosome signals (products of stem cell local incorporation and differentiation (Months 7-12).
Performed by MR

Performance of this step is not possible at this stage.

Key Research Accomplishments:

None

Reportable Outcomes

None. Any paper that will stem from this research in the future will include acknowledgement of this grant of the DoD.

Conclusions:

There is compelling evidence that blood vessels are derived from circulating precursors in the cancer microenvironment, and that these have distinct physiological and immunological features that modify the cancer pathology. We are interested in identifying all cancer stromal components, derived from circulating precursors. Our hypothesis is that the wound healing nature of the cancer “invites” cells that contribute to the overall self renewal potential of the cancer tissue. This would provide anticancer research new avenues to attack this disease. Unfortunately, our model is facing (with the exception of DoD, which were great in support and patience) an overall objection to our hypothesis, and need to acquire more basic research evidence before we are allowed access to the valuable human specimens we need, to make our point.

Table of content for the Appendices:

- Page 7-8: Current application.
- Page 9-19: kCONFAB application
- Page 20: kCONFAB committee questions.
- Page 21-24: Our answers to the committee.
- Page 25: The final answer.
- Page 26: Tissue Bank approval.

Current animal model study:

In common with stem cells in normal healthy tissues whose properties are regulated by their interactions with a specific cellular microenvironment within the host tissue (*stem cell niche*), the behaviour of tumour cells is also strongly influenced by their interactions with the surrounding tissue microenvironment. Adjacent to the tumor cells are host cells that are not transformed, but despite appearing to be normal blood vessel or connective tissue cells, they participate in tumor progression as assessed phenotypically[6, 7] or by gene expression profiling [8]. The nature of these interactions remains poorly understood as does the identity and origin of the tumor associated vascular and stromal elements. The mechanism likely involves local exchange of signals between tumor and stromal cells, and/or recruitment of precursor cells from the circulation, which develop as an independent, “cancer-specific” lineage. While it has been demonstrated that blood vessel endothelial cells are derived in part from circulating precursor cells[6, 9], no such evidence exists for carcinoma-associated fibroblasts (CAFs). We have shown that normal breast fibroblasts do **not** convert to CAFs during co-growth with breast tumor cells in mice. *Here we propose to investigate whether CAFs, like tumor blood vessels, are recruited into the tumor from circulating precursors.* The overall aim of the project is to determine whether bone marrow derived fibroblast precursors are recruited to primary tumors and whether these cells contribute to tumor progression and distant metastasis.

The Specific Aims are as follows:

1. To determine whether bone marrow derived fibroblast precursor cells migrate to the site of primary tumors and become CAF.
2. To isolate bone marrow derived fibroblast precursors from primary tumors and test their ability to promote tumor growth and distant metastasis.

Research Design: Mesenchymal precursor cells (MPC) will be isolated from Bones, as published previously [10] either from ROSA/lacZ mice (expressing β Gal) or from Granzyme M KO mice (expressing the neo^R selectable marker). Granzyme M KO mice will be used as a source of neo tagged MPC that are unlikely to have a phenotype associated with the lack of granzyme M since this protease has a localization restricted to NK lymphocytes. The MPC isolation method is established in the laboratory of our collaborator. These MPC will be introduced via an intrafemoral injection into 6 week old female C57BL/6 mice, resulting in mice populated with mesenchymal precursor cells that can be identified either by immunohistochemistry (IHC) using antibodies against β Gal or by growth in the presence of lethal doses of Geneticin (G418). After 30 days, EO771 cells, a C57BL/6 derived metastatic mammary tumor line will be inoculated into the fourth mammary gland of the recipient mice. Following primary tumor growth and metastasis, primarily to lung over a period of 3-4 weeks, **the contribution of MPC to fibroblasts residing adjacent to breast cancer cells will be assessed via three strategies:** **I.** Genomic DNA isolated from the primary tumor and several organs, including lung, lymph nodes and liver, will be screened for the presence of the marker (β Gal or neo^R) by quantitative PCR as a rapid screen for tissues positive for the transplanted MPC. **II.** The primary tumor and qPCR positive tissues will be examined by IHC for the presence of β Gal. Co-localization of β Gal and fibroblast markers such as FSP, Col 1 and α SMA and the lack of leukocyte markers (CD45) and endothelial cell markers (CD31) will confirm the fibroblastic lineage of these MPC derived cells in the primary tumor and metastatic nodules. **III.** Primary tumors grown in mice transplanted with the neo^R MPC will be excised, disaggregated and grown in culture in the presence of G418. The adherent G418 resistant cells recovered after 2-3 weeks in culture will be assayed for mesenchymal colony forming potential in the presence of G418 and the relevant media to provide precise quantitation of the incidence of clonogenic MPC/organ[10]. **IV.** Isolated neo^R MPC-derived cells (as in III.) will be co-transplanted with fresh EO771 cells into naïve C57BL/6 mice to assess their impact on the rate of growth and the metastatic capacity of these mammary tumors, compared to EO771 cells co-transplanted with normal mammary fibroblasts or EO771 transplanted alone. The CAF phenotype will be measured by acceleration of tumor formation by the EO771 breast cancer lines[6]. Collectively these data will provide independent measure for the frequency by which the fibroblasts that are adjacent to cancer cells, and exhibit altered phenotype and expression patterns, are derived from circulating precursors.

References:

1. Foulkes, W.D., *BRCA1 functions as a breast stem cell regulator*. J Med Genet, 2004. **41**(1): p. 1-5.
2. Smalley, M. and A. Ashworth, *Stem cells and breast cancer: A field in transit*. Nat Rev Cancer, 2003.

- 3(11): p. 832-44.
3. Evans, P.C., et al., *Long-term fetal microchimerism in peripheral blood mononuclear cell subsets in healthy women and women with scleroderma*. Blood, 1999. **93**(6): p. 2033-7.
4. Petersen, B.E., et al., *Bone marrow as a potential source of hepatic oval cells*. Science, 1999. **284**(5417): p. 1168-70.
5. Srivatsa, B., et al., *Microchimerism of presumed fetal origin in thyroid specimens from women: a case-control study*. Lancet, 2001. **358**(9298): p. 2034-8.
6. Orimo, A., et al., *Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion*. Cell, 2005. **121**(3): p. 335-48.
7. Olumi, A.F., et al., *Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium*. Cancer Res, 1999. **59**(19): p. 5002-11.
8. St Croix, B., et al., *Genes expressed in human tumor endothelium [see comments]*. Science, 2000. **289**(5482): p. 1197-202.
9. Yang, L., et al., *Expansion of myeloid immune suppressor Gr⁺CD11b⁺ cells in tumor-bearing host directly promotes tumor angiogenesis*. Cancer Cell, 2004. **6**(4): p. 409-21.
10. Short, B., et al., *Mesenchymal stem cells*. Arch Med Res, 2003. **34**(6): p. 565-71.

Evidence that supports the existence of a tumor stem cell for breast cancer has given fresh impetus to the search for an adult mammary epithelial stem cell in the normal breast (Smalley and Ashworth, *Nat Rev Cancer*, '03). Such a cell might be responsible for routine tissue renewal and the massive expansion in epithelial tissue that the breast undergoes during pregnancy, and might be the cell of origin of most, if not all, breast tumors. Alternatively, the cancer cell may recruit stem cells to specific stromal cell populations, thereby providing vital milieu for the cancer development. This grant proposal offers a novel method to identify, which cells in the tumor (malignant or stroma), if any, have originated from the body's stem cell pool. Such a cell may present new opportunities for the development of novel therapeutic and prophylactic strategies for treating breast cancer.

Embryonic stem cells augment the mother's stem cell pool (Evans, Lambert et al., *Blood*, '99). Transplanted or chimeric Y chromosome-bearing stem cells behave as do the mothers own: aggregating, proliferating, and differentiating (Petersen, Bowen et al., *Science*, '99; Srivatsa, Srivatsa et al., *Lancet*, '01). In our proposal, the Y-bearing cells are assumed to be no more than a marker for stem cell-derived somatic cells: whether they have in any way differentiated; into what cell line; is there any evidence of bias in relation to the type of cells they generate? We argue that any positive results produced by our investigations must be viewed in the context of the patient's overall stem cell activity.

Our Microarray study with Ovarian cancer (AOCS/CDRP000109), has shown that nine out of eighty four female cancer tissues, contained Y-chromosome sequences in their mRNA. This implies that these females had microchimerism stem cells incorporated into their cancer tissue (Cha, Khosrotehrani et al., *Obstet Gynecol*, '03). The Australian consortium for research into familial breast cancer (kConFab, www.kconfab.org) has collected 1455 bloods for females affected with breast cancer. 995 of those have had male babies. Of those, 85 had the baby within five years of initial diagnosis, which offers to assess whether the cancer cell originated from a stem cell (Smalley and Ashworth, *Nat Rev Cancer*, '03). Assuming 10% of the patients are positive, we would have more than ninety positive sections. Blood DNA from these patients is available for research purposes. Those patients have given open consent for use of their bio-specimens for any project approved by a scientific board.

1. Using PCR we will test for Y chromosome in blood DNA. Female breast cancer patients with Y-chromosome in their blood DNA will be selected for further analysis. A corresponding control group from patients who didn't have male babies will be analysed in parallel. Invasive, DCIS and neighbouring normal tissue blocks (Formalin fixed, paraffin embedded) will be taken for further investigation.
2. Those samples will have histological sections examined by fluorescent in situ hybridisation (McKay, Murray et al., *Mol Pathol*, '97), to find those cells carrying Y chromosome.
3. The Y-chromosome positive sections will be double stained with cell lineage specific markers, such as Cytokeratin, Factor VIII, Vimentin, CD11, CD3 and GCDFP (myoepithelial cell marker).

- Cha, D., K. Khosrotehrani, et al. (2003). "Cervical cancer and microchimerism." Obstet Gynecol **102**(4): 774-81.
- Evans, P. C., N. Lambert, et al. (1999). "Long-term fetal microchimerism in peripheral blood mononuclear cell subsets in healthy women and women with scleroderma." Blood **93**(6): 2033-7.
- McKay, J. A., G. I. Murray, et al. (1997). "Amplification of fluorescent in situ hybridisation signals in formalin fixed paraffin wax embedded sections of colon tumour using biotinylated tyramide." Mol Pathol **50**(6): 322-5.
- Petersen, B. E., W. C. Bowen, et al. (1999). "Bone marrow as a potential source of hepatic oval cells." Science **284**(5417): 1168-70.
- Smalley, M. and A. Ashworth (2003). "Stem cells and breast cancer: A field in transit." Nat Rev Cancer **3**(11): 832-44.
- Srivatsa, B., S. Srivatsa, et al. (2001). "Microchimerism of presumed fetal origin in thyroid specimens from women: a case-control study." Lancet **358**(9298): 2034-8.

CHECKLIST OF MATERIAL REQUIRED AS PART OF FULL AND PILOT APPLICATIONS FOR BIOSPECIMENS AND DATA

- π Scientific proposal (up to six pages) including aims, hypotheses to be tested, significance, background, research plan with details of methods to be used and references. This should include the rationale for number and amount of samples requested, including considerations of statistical analysis and statistical power. Less than one page is required for a pilot project.
- π List of biological material requested including the type of sample, the number of samples, and the amount of sample.
- π Completed KConFaB Data Request Form (see attached)
- π Evidence of ethical clearance for the project including copies of approved institutional human research ethics applications and all correspondence with the human research ethics committee. Where applicable this must be provided from each of the participating institutions.
- π Evidence of approval for a grant application that has already undergone peer review by a funding agency. Wherever possible, KConFab would appreciate receiving copies of the referees' reports. This is not required for pilot projects.
- π Names of three suitable referees for grants that have not already undergone peer review or for which peer review from an external granting body is not pending. Applicants may also nominate people whom they do not wish to review the application. This is not required for pilot projects.
- π Information on the resources available to conduct the research (including source of funds, personnel, and maintenance).
- π Publications of the Chief Investigator(s) for the last five years.
- π Suggested timeline for the project including batch sizes and reasonable monitoring procedures for KConFab to use, and predicted time for submitting data to the KConFab database. Indicate when the first request for data and biological specimens will be made following approval of the project. Successive batches may not be shipped until data from the previous batch(es) have been received at the central database according to the agreed schedule. In the event that the data are incomplete or are otherwise unacceptable, KConFab reserves the right to withhold further shipments of material.
- π Suggested protocol for shipping, including (a) mode of shipping, (b) address for shipping, and (c) suggested arrangement for payment of shipping.
- π An outline of consulting agreements, collaborations and research projects between investigators named on the application and commercial organisations.

SCIENTIFIC PROPOSAL

Project Title: “ANALYSIS OF THE CONTRIBUTION OF STEM CELLS TO BREAST CANCER USING MICROCHIMERISM-BASED Y-CHROMOSOME STAINS AND HISTOPATHOLOGY ”

1.0 Aims:

To utilize microchimerism to monitor circulating stem cell incorporation into breast cancer tissue, we suggest to follow three steps:

- **PCR screen from blood DNA to detect Y-chromosome positive cells circulating in mothers' blood.**
- **To hybridize Y-positive probe (In Situ Hybridization) to tissue sections of the patients identified in aim 1.**
- **To accompany these stains with markers for typical cell types, which will be used for histo-pathological analysis.**

2.0 Hypotheses

The Y-chromosome in this project is merely a “tracker” of circulating stem cells, capable of differentiating into different cell lineages in all or most organs of the body, including epithelial, endothelial, macrophage, or fibroblast cells. The breast organ should not be an exception to other organs. In thyroid, cervix, skin, intestine and gall bladder, 14-60% of Y-chromosome staining cells in females are cytokeratin positive. The measured frequency of these cells in these adult mother tissues is approximately 0.7%. This means that in a tissue section with 1000 cells in the field, we should be able to see more than ten such cells. In addition, we show in Figure 1, similar signal, found in stomach and ovarian cancer tissues. Our frequency of occurrence is way higher than recorded, suggesting that if the ISH was performed on the proper mRNA sequence, rather than the DNA, more cells would have been found as XY+. We hypothesize that due to the altered microenvironment of cancer, which potentially improves stem cell niche (for the cancer stem cell), circulating naïve stem cells will also be affected, and their frequency, and nature of incorporation might be altered in the cancer tissue over a neighbouring tissue.

Objective: To identify, which cells in the tumor (malignant or stroma), if any, have originated from the body's circulating stem cell pool. Such a cell may present new opportunities for the development of novel therapeutic and prophylactic strategies for treating breast cancer.

Expected outcome: Sporadic stain of cancer cells with Y-chromosome would imply that some cancer cells are not descendents of founder cell and that transformation in the tumor microenvironment is ongoing. Stain of stromal cells, say blood vessel, would imply local differentiation of circulating stem cell as part of angiogenesis (rather than local blood vessel recruitment).

Possible outcomes are:

Outcome 1. If Y-chromosome stains a subset of the otherwise indistinguishable cancer cells, this will challenge the currently accepted dogma of that cancer arises from a founder clone, and descendents of these founder constitute all cancer cell in the advanced tumour. Rather, it would suggest that the the cancer microenvironment is capable of instructing de novo malignant transformation of neighbouring stem cells or epithelial cells.

Outcome 2. Y-chromosome inclusion in the cancer specimen exceeds that of the matched normal area of the slide, for a defined cell population (*i.e.* immune cells or blood vessel). This would mean that fundamental processes in cancer, such as angiogenesis, are mediated through the selective recruitment of a circulating pluri-potent stem cell rather than local communication with the residential members of this cell population.

Outcome 3. Y-chromosome carrier cells are concentrated in the tumour mass in an undifferentiated cell population that can be stained for stem cell markers, such as CD24 low, CD34+, CD133+. This would imply that cancer microenvironment is a stem cell niche, and circulating pluri-potent cells are “happy” in this environment. This in turn would direct further research to abrogating this aspect of cancer in the course of future therapy.

3.0 Significance

Strong evidence in a few types of leukemia, breast cancer, and colon cancer, indicate that only a small number of cells are actually capable of long term growth, and exhibit the ability to colonize new tissues, while most cancer cells, much like any other adult somatic cells, are destined to die. If this is true, then most common treatment modalities, and more importantly, the way we measure success (through markers of the total mass of the tumour cells), are irrelevant! Future treatments, which hopefully will rectify the high frequency of relapse in the patients, will directly target the cancer stem cell, rather than those cancer cell destined to die anyway. In particular, BrCa1 mutations were speculated to affect breast stem cell development, making the kCONFAB cohort an ideal one to address these questions. All the outcomes described above, could potentially be different between the BrCa1+ patients, and the rest of kCONFAB, which would further support the suggestion that BrCa1+ patients have altered management of stem cells in their breasts. This study hardly relies on preexisting knowledge or hypothesis. It is mainly an exploratory study, which allows the nature of the samples to dictate the conclusion. The microchimerism is merely an opportunistic experimental design, allowing for an unbiased controlled measure of circulating stem cell incorporation in adult human tissues that would otherwise be impossible from technical and ethical points of view. Therefore, the significance of this study is hard to predict, and will depend on the observations obtained. It has an enormous contribution to a growing interest in the mystery of what cells in the tumour tissue originate from circulating pluripotent cells, and as such, may help direct novel therapies to cancer, based on essential regenerative potential in the tumour tissue. Both in the ovarian and gastric samples, we find a weak association between Y-chromosome signal and patient outcome. Although this observation is not confirmed, and will not be possible to address with kCONFAB samples, it may point to a possible correlation between how “attractive” a cancer tissue is for a stem cell (of any sort), and its ability to regenerate after treatment modalities.

4.0 Background

Most cells in our body have limited life span. Evidence that supports the existence of a tumor stem cell for breast cancer has given fresh impetus to the search for an adult mammary epithelial stem cell in the normal breast. Such stem cells, licensed for extended tissue repopulation, are proposed to be necessary for cancer tissue maintenance. Compelling data argues, that the cells neighboring the cancer cell, collectively called stroma, are partially responsible for the cancer phenotype and etiology. Therefore, the malignant epithelial cell may recruit stem cells to specific stromal cell populations, thereby providing vital milieu for the cancer development. Embryonic stem cells augment the mother's stem cell pool. Transplanted or chimeric Y chromosome-bearing stem cells behave as do the mothers own: proliferating, differentiating and incorporating into adult

epithelial tissue. We view this phenomenon termed Microchimerism, as an opportunity to address stem cell incorporation in cancer tissue. We will access blood samples from 995 breast cancer patients, who had male babies, screen for Y-chromosome DNA and focus on positive patients. Assuming 10% of the patients are micro-chimeric, we would have more than ninety positive patients. Looking at sections of the corresponding cancers we will ask which cells in the tumor (malignant or stroma), if any, have originated from the body's circulating stem cell pool, using the Y-chromosome in micro-chimeric mothers. Such a cell may present new opportunities for the development of novel therapeutic and prophylactic strategies for treating breast cancer.

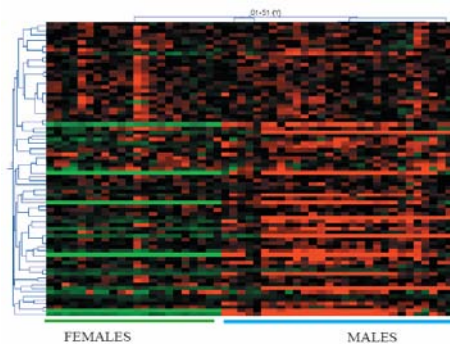
BRCA1 cancers have been proposed to arise from a stem cell deregulation¹. Microchimerism offers a unique opportunity to assess that proposal.

Evidence that supports the existence of a tumours stem cell for breast cancer has given fresh impetus to the search for an adult mammary epithelial stem cell in the normal breast². Such a cell might be responsible for routine tissue renewal and the massive expansion in epithelial tissue that the breast undergoes during pregnancy, and might be the cell of origin of most, if not all, breast tumours. Alternatively, the malignant epithelial cell may recruit stem cells to specific stromal cell populations, thereby providing vital milieu for the cancer development. This grant proposal offers a novel method to identify, which cells in the tumours (malignant or stroma), if any, have originated from the body's stem cell pool. Such a cell may present new opportunities for the development of novel therapeutic and prophylactic strategies for treating breast cancer. Embryonic stem cells augment the mother's stem

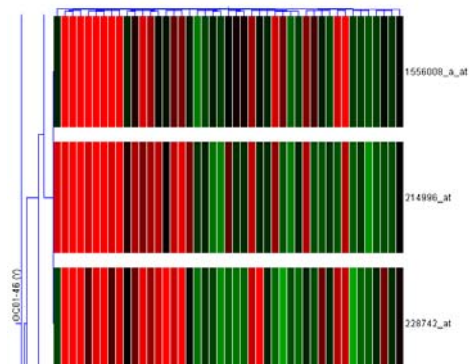
cell pool³. Transplanted or chimeric Y chromosome-bearing stem cells behave as do the mothers own: proliferating, differentiating and incorporating into adult epithelial tissue^{4, 5}. In our proposal, the Y-bearing cells are assumed to be no more than a marker for stem cell-derived somatic cells: whether they have in any way differentiated; into what cell line; is there any evidence of bias in relation to the type of cells they generate? We argue that any positive results produced by our investigations must be viewed in the context of the patient's overall stem cell activity.

4.0 Research Plan

1. **To identify microchimerism in kCONFAB patients, that had male babies.** We have designed three TaqMan® primer pairs that amplify Y-chromosome areas that are elevated in the female samples in Figure 1. Using blast, we confirmed that these reagents would not hybridize to any other sequences in the genome. Using these PCR primers, we will test for Y-chromosome in blood DNA. First, we will confirm that the primers do not amplify DNA from females who had no babies, and do amplify DNA from males. The specific reagent will then be used to screen the 995 female blood DNA (Buffy coat), using our Biomac® robot, we will distribute the primers into 384 well plates. The screen will require three plates per primer pair. This means that when



Y-Chromosome signals in Gastric Cancer specimens (above), and Ovarian Cancer Specimens (below).



the assay is running, the screen should take no more than three days (two hours a plate run). Female breast cancer patients with Y-chromosome in their blood DNA will be selected for further analysis. The real time PCR method is quantitative, and we expect a gradient of abundance of the Y-chromosome signal in the blood. We will rank the processing of these samples according to abundance of Y-chromosome signal in the blood DNA, which we hope, will correlate with frequency of Y-chromosome staining on the tissue sections. A corresponding control group from patients who only had female babies will be analysed in parallel. For positive control we'll use 20 male breast cancers. Invasive, DCIS, LCIS and neighbouring normal tissue blocks (Formalin fixed, paraffin embedded) will be supplied by kConFab for further investigation.

2. **To identify the cells that contain Y-chromosome (ostensibly products of circulating stem cell incorporating in the tissue as part of tissue regeneration process):** The microchimeric mothers, as well as the control samples will have histological sections examined by Fluorescent In Situ Hybridization⁷, to find those cells carrying Y-chromosome. We will use both BAC chromosome clones which detect the Y-chromosome DNA, as well as three mRNA probes. While the former does not depend on the gene being expressed, and thus is more likely to produce a signal where there is a male cell, the latter signal is spread throughout the cell cytoplasm, and therefore will give signal in multiple sections of the cell. Our microarray results allowed us to identify Y-chromosome sequences that are expressed in female bodies, as opposed to sequences that are not (Figure 1). In situ hybridizations will be performed by Christina Restall in R. Anderson lab, who routinely use the method for their purposes. Initial inspection of the cells that stain for Y-chromosome should tell us what cells might those be. This part of the work will be performed by Melissa Robbie, a gynaecological Histopathologist doing her sabbatical year in our lab.
3. **To identify the cell lineages that incorporate the circulating stem cells, via the Y-chromosome signal, in normal and malignant environments:** The Y-chromosome positive sections will be double stained with cell lineage specific markers, such as Cytokeratin, Factor VIII, CD11, CD3 and GCDFP (myoepithelial cell marker). Matching serial sections will be stained with H&E. We will explore the possibility that following ISH, the section will further be processed in the pathology lab, using their standard immunohistochemistry procedures. If this doesn't work (likely), we will have to perform double staining in our lab. Christinal Restall has already stained tissue for three of these markers, to confirm feasibility.

5.0 Rational for numbers requested

Our Microarray study with ovarian cancer (AOCS/CDRP000109) has shown that nine out of eighty four female cancer tissues contained Y-chromosome sequences in their mRNA. This implies that these females had microchimerism stem cells incorporated into their cancer tissue⁶. This figure of 10% does not take into account the number of women that had a male baby before, which means that when addressing mothers of male babies specifically, an even higher frequency of microchimerism is expected. The Australian consortium for research into familial breast cancer (kConFab, www.kconfab.org) has

collected 1455 bloods for females affected with breast cancer. 995 of those have had male babies. Of those, 85 had their sole male infant within five years prior to initial diagnosis, which implies that the Y-chromosome, if present positive cells are not the founder cancer cell ².

Assuming 10% (a cautious assessment, based on the microarray results) of the patients are positive, we would have more than ninety positive sections. More than half the positive cells should be visible in 5 μ sections, assuming nuclear diameter is ~7 μ . Blood DNA from these patients is available for research purposes. Those patients have given open consent for use of their bio-specimens for any project approved by a scientific board.

6.0 ETHICAL CONSIDERATIONS

At this stage we do not foresee any controversial aspect of our work for all ethical purposes.

7.0 REFERENCES

Biological Material Requested

1. 1035 Buffy coat blood DNA (2 μ gr each).
2. ~100 7 μ m tissue sections from formalin fixed paraffin embedded tissues of the breast cancer specimen from microchimeric females found with the first requested material.
3. ~10 repeats of highly staining cases of requested material 2, for multiple co-staining procedures.

Data Request form

Attached

Peer Reviewed funding:

This work is funded by the US Army DoD breast cancer program concept award BC023752.

Publications of the CIs for the last 5 years

1. Tothill, R.W., et al., *An expression-based site of origin diagnostic method designed for clinical application to cancer of unknown origin*. Can Res, 2005. **In Press**.
2. Boussioutas, A., et al., *Distinctive patterns of gene expression in premalignant gastric mucosa and gastric cancer*. Cancer Res, 2003. **63**(10): p. 2569-77.
3. Boussioutas, A. and I. Haviv, *Current and potential uses for DNA microarrays in transplantation medicine: lessons from other disciplines*. Tissue Antigens, 2003. **62**(2): p. 93-103.
4. Boussioutas, A., et al., *Distinctive Patterns of Gene Expression in Premalignant Gastric Mucosa and Gastric Cancer*. Cancer Res, 2003. **63**(10): p. 2569-2577.

5. Weston, G.C., I. Haviv, and P.A. Rogers, *Microarray analysis of VEGF-responsive genes in myometrial endothelial cells*. Mol Hum Reprod, 2002. **8**(9): p. 855-63.
6. Haviv, I. and I.G. Campbell, *DNA microarrays for assessing ovarian cancer gene expression*. Mol Cell Endocrinol, 2002. **191**(1): p. 121-6.
7. Dickins, R.A., et al., *The ubiquitin ligase component Siah1a is required for completion of meiosis I in male mice*. Mol Cell Biol, 2002. **22**(7): p. 2294-303.
8. Haviv, I., Y. Matza, and Y. Shaul, *pX, the HBV-encoded coactivator, suppresses the phenotypes of TBP and TAFII250 mutants*. Genes Dev, 1998. **12**(8): p. 1217-26.
9. Haviv, I., et al., *Hepatitis B virus pX targets TFIIIB in transcription coactivation*. Mol Cell Biol, 1998. **18**(3): p. 1562-9.

Timeline

We hope to begin ISH of aim 2 within a month of approval of the project. This part of the project will continue for five months. The third part of the project is dependent on the pathology expert and is hard to predict.

Protocol for shipping

Not relevant.

Consulting agreements, collaborations and research projects between the applicants and commercial organizations

none

Kconfab Data Request Form
9656 1457

Please fax completed form to Heather Thorne

Please read the notes on the front page before completing details of your request:

Name: Izhak Haviv, Melissa J Robbie and Harry Corbett

Organisation: Peter MacCallum Cancer Centre

Kconfab Project: "ANALYSIS OF THE CONTRIBUTION OF STEM CELLS TO
BREAST CANCER USING MICROCHIMERISM-BASED Y-CHROMOSOME
STAINS AND HISTOPATHOLOGY "

Date of request: 11/03/2005 Date data required: 3/18/2005

Your contact details

E-mail: izhak.haviv@petermac.org

Fax: 03-96561411

Phone: 03-96561287

Names of other contact people for this project

Name Harry R. Corbett

E-mail: harryc@myaccess.com.au

Phone: 03-97206800

Name Melissa J Robbie

E-mail: melissa.robbie@petermac.org

Phone: 03-96561284

Please enter full details of your request here:

A unique code number for each patient (UPN, UFN, No names or other personal information!)

Data dump, family tree

Questionnaire A2 (DOB, gender of baby), B2, H1-9

For each sample:

Estrogen Receptor status

Lymph node, size, and grade status

Initial pathology report

In which format would you like your data, e.g., MS Excel spreadsheet, Progeny database:

Microsoft Excel spreadsheet.

**A data request number and contact name will be sent to you as soon as possible.
Please email the contact person if you need to clarify details of your request.**

kConFab Subcommittees

kConFab number:47/03-04

Applicants: Izhak Haviv

Date: 1 April 2005

- 1) There is no obvious relevance to familial cancer. We advise carrying out studies on sporadic breast cancers initially, and if these prove to be interesting a pilot study on kConFab cases may be considered. The stated rationale relating to BRCA1 is irrelevant given that BRCA1-positive individuals are not being selected and no information about mutation status has even been requested?
- 2) The application was very difficult to evaluate as the preliminary data were uninterpretable, the hypotheses unclear and the arguments regarding feasibility largely unsupported. If a resubmission were made, it would be helpful if these issues were considered.
- 3) The amount of material requested is high and also for a number of reasons the request would be very labour intensive for kConFab. Evidence from a pilot study, and justification for why a lower amount could not be used would be helpful in addressing the resource issues and are recommended if a resubmission is made.
- 4) The term 'stem cell' is not defined and is inconsistently applied. In particular reference to the 'stem cell pool' is confusing.
- 5) In normal tissues, 'stem cells' are cells capable of self renewal that give rise to a range of differentiated cells types. These have been identified and characterised principally in the haemopoietic system. In the normal breast stem cells are inferred but have not been conclusively identified. Cancer 'stem cells' are tumour cells capable of self renewal.
- 7) The material requested is unclear. How many tumour sections per positive case are required? The application appears to specify 100 which is excessive.
- 8) Please clarify the specific hypotheses relating to breast cancer susceptibility and tumour formation that will be tested by these experiments?

kConfab Subcommittees

kConfab number:47/03-04

Applicants: Izhak Haviv

Date: 1 April 2005

Dear Heather and kConfab committee members,

Thank you for your prompt and professional feedback; I hope this letter answers your issues. Please let me know if I need to answer in a different format, or if my response is not satisfactory in any way. It must be stated at the outset that the financial support of the US DoD for this project was contingent upon our access to kConfab samples and our application to the US DoD carried with it a letter from kConfab expressing the view that the project was compliant with kConfab objectives. Any denial of access to kConfab samples thus puts us in an awkward situation.

1) The is no obvious relevance to familial cancer. We advise carrying out studies on sporadic breast cancers initially, and if these prove to be interesting a pilot study on kConfab cases may be considered. The stated rationale relating to BRCA1 is irrelevant given that BRCA1-positive individuals are not being selected and no information about mutation status has even been requested?

We chose kConfab for three reasons:

- The samples are from a population based tissue collection that is perfectly annotated and thus allows an effective screen for microchimerism. Our screen is a simple assay (up to a thousand samples may be screened in one day) and will use only a minute amount of kConfab material (50 ng blood DNA per sample, and we are happy to pay for the sample processing). Whereas we now acknowledge that these numbers will not be made available to us, we are happy to access a smaller number of more carefully selected patients.
- The kConfab questionnaire is unique in its richness of information, recording data on children's date of birth and gender, which are of paramount importance for this study.
- The involvement of BrCa1 in stem cell deregulation. Since then, BrCa2 mutation has also been implicated in stem cell deregulation and epithelial cancer (Hay et al., Oncogene, 2005).
-

While our first reason does not necessarily involve familial cancer mechanisms, the collection of multiple cancers within a family offers information that is of paramount importance to us. For example, it is unclear what dictates the efficiency of microchimerism. By following family members, it is possible that a role for the genomic background of the mother will become clear.

We did not ask for BrCa1 mutation alone because we do not know if this trait will be shared by all susceptible germ line genes, as BrCa2 is now implicated as well. Also, one can not make any statement on the role of BrCa1, unless samples from non-BrCa1 are compared. We did make a mistake not to ask for BrCa1 status; we had thought it be better run as a blind experiment. We now wish to obtain mutation status of the individuals. We also ask to minimize the number of samples to the ~150 cases that are BrCa1 mutants, and an equivalent number from controls/non-BrCa1. From those we ask for 50 ng of blood DNA. We expect that about thirty of those will have Y-chromosome signals. Of those thirty, we ask for a couple of sections from the tumour block, where possible. With this information we will design a model that predicts how many samples we need from each tumour type, and assess whether we need more samples, through a future application.

2) The application was very difficult to evaluate as the preliminary data were uninterpretable, the hypotheses unclear and the arguments regarding feasibility largely unsupported. If a resubmission were made, it would be helpful if these issues were considered.

This study hardly relies on pre-existing knowledge (preliminary results) or hypothesis. The project does depend on the phenomenon of microchimerism which is well documented in the literature (Idilman et al., Transplantation, 2004; Khosrotehrani et al., Jama, 2004; Mengel et al., J Am Soc Nephrol, 2004). It is mainly an exploratory study, which allows the nature of the samples to dictate the conclusion. The basic hypothesis is that organ resident Y-chromosome positive cells in mothers must have made their way from trophoblast tissue to ovary, stomach, breast or any other tissue, in the form of a pluripotent circulating cell (Sato et al., Transplant Proc, 2005), normally found in adult bone marrow. The notion that foetal cells find their way to the maternal breast (for example) by any other pathway is simply not tenable. When these cells differentiate in the target organs, they can still be tracked through the Y-chromosome. That is what we need to assume for the study to succeed.

The mother blood DNA is an easy assay, allowing high throughput screening. Our focus of interest involves the possibility that the differentiation status, or the type of cell carrying Y-chromosome in the cancer tissue, differs from that found in an equivalent volume of normal tissue. This would imply that the cancer environment recruits the potential pluripotent circulating cell, to incorporate into the tumour a cell function needed for cancer development, such as blood vessel, or additional deformed epithelial cells (which are obviously not derivatives of the founder cell of the tumour as indicated by the Y-chromosome). At least for blood vessels, unfortunately, we know there is already a paper (Yang et al., Cancer Cell, 2004), describing just that! If we are to contribute original information to this field we need to make a move.

We readily accept that kConfab's primary aim is to shed light onto the mechanisms of cancer promotion by the BrCa mutated genes. These large genes have already been implicated in multiple mechanisms. We are now suggesting a unique opportunity to address the possibility that stem cell deregulation and organ regeneration is abrogated in BrCa mutant patients. We are aware that we need sporadic cases for comparison, and will utilise PMCC tissue bank samples to serve as controls.

3) The amount of material requested is high and also for a number of reasons the request would be very labour intensive for kConfab. Evidence from a pilot study, and justification for why a lower amount could not be used would be helpful in addressing the resource issues and are recommended if a resubmission is made.

The amount of DNA required is minimal, i.e. enough for four real time PCR reactions (10 ng each). We were hoping to access a large number of blood DNA samples, as only ~15% of kConfab patients are BrCa1 and of those only 10% would be microchimeric (based on our preliminary results, as well as eight current publications in the field). Of 995 cases this would lead to a little over ten cases to section and stain for Y- chromosome, against an equivalent number of controls. We think that is the size of a pilot. If a consistent bias in presence of Y-chromosome cells is observed and we feel the information is of the quality to warrant publication in a high impact factor journal, then we will obviously need the appropriate number of samples as dictated by statistical guidelines. Ten sections, we suggest, is a good start.

4) The term 'stem cell' is not defined and is inconsistently applied. In particular reference to the 'stem cell pool' is confusing.

All cell populations in the mouse mammary gland will be found to be tagged with a specific marker gene (viral or LacZ, respectively), if this marker is introduced into mice in the form of a mammary gland resident stem cell (Kordon et al., Development, 2003) or bone marrow cells (Welm et al., Cell Prolif, 2003). These two publications suggest that organ regeneration is fed from both organ resident stem cell as well as bone marrow progenitors. The strongest in vivo evidence for circulating stem cells contributing to epithelial cells in adult organ regeneration comes from microchimerism (Idilman et al., Transplantation, 2004; Khosrotehrani et al., Jama, 2004; Mengel et al., J Am Soc Nephrol, 2004). Two papers suggest that breast cancer is populated by microchimerism based foetally-derived cells that involve the immune response to the cancer (Bishop, Haematologica, 2004; Davies, Thyroid, 1999).

5) In normal tissues, 'stem cells' are cells capable of self renewal that give rise to a range of differentiated cells types. These have been identified and characterised principally in the haemopoietic system. In the normal breast stem cells are inferred but have not been conclusively identified. Cancer 'stem cells' are tumour cells capable of self renewal.

We have no interest in the breast cancer stem cell. The rare frequency expected for such an event makes it unlikely for us to access sufficient numbers of those cancers. That is why we wish to target mothers that have had male babies within the last five years. In such a scenario, assuming that the cancer founder population predates conception, the contribution of foetal cells to a breast cancer stem cell is unlikely. The finely tuned focus of I. Haviv's vast body of internationally recognised work involves tumour-stroma interactions that modify the clinical features of cancer, such as angiogenesis. This study utilises and extends work already bedded down. The stem cell contribution we are mainly interested in involves the cancer cell's neighbours; we wish to determine whether such cells, epithelial, fibroblastic, endothelial, or otherwise, arise from a circulating cell and thus establish in greater detail the reason for the well established presence of microchimeric cells in the cancer process.

7) The material requested is unclear. How many tumour sections per positive case are required? The application appears to specify 100 which is excessive.

Two to five sections are requested per patient. We expected a hundred patients to make it through the initial screen. We now expect approximately thirty.

8) Please clarify the specific hypotheses relating to breast cancer susceptibility and tumour formation that will be tested by these experiments?

Cells that populate and contribute to normal and injured tissue are partially derived from circulating cells that migrate into adult organs via the blood stream and become differentiated in the organ as needed. This process, generally referred to as a stem cell niche, is suggested to be abrogated by BrCa mutations. Comparing cancerous tissue from different microchimeric mothers (BrCa1, BrCa2, and BrCaX) with neighbouring non-tumour tissue may reveal such an abrogation, through a bias in the identity of Y-chromosome positive cells. For example, it is possible that cancer blood vessels (Yang et al., Cancer Cell, 2004) have different gene expression patterns and physiological properties, because they are actually derived from a distinct biological control mechanism.

Bishop, M. R. (2004). Allogeneic hematopoietic stem cell transplantation for metastatic breast cancer. *Haematologica* 89, 599-605.

Davies, T. F. (1999). The thyroid immunology of the postpartum period. *Thyroid* 9, 675-684.

Hay, T., Patrick, T., Winton, D., Sansom, O. J., and Clarke, A. R. (2005). Brca2 deficiency in the murine small intestine sensitizes to p53-dependent apoptosis and leads to the spontaneous deletion of stem cells. *Oncogene*.

Idilman, R., Erden, E., Kuzu, I., Ersoz, S., Karasu, Z., Karayalcin, K., Yuce, G., Tokat, Y., Sahin, Y., Tukun, A., *et al.* (2004). Recipient-derived hepatocytes in sex-mismatched liver allografts after liver transplantation: early versus late transplant biopsies. *Transplantation* 78, 1647-1652.

Khosrotehrani, K., Johnson, K. L., Cha, D. H., Salomon, R. N., and Bianchi, D. W. (2004). Transfer of fetal cells with multilineage potential to maternal tissue. *Jama* 292, 75-80.

Mengel, M., Jonigk, D., Marwedel, M., Kleeberger, W., Bredt, M., Bock, O., Lehmann, U., Gwinner, W., Haller, H., and Kreipe, H. (2004). Tubular chimerism occurs regularly in renal allografts and is not correlated to outcome. *J Am Soc Nephrol* 15, 978-986.

Sato, Y., Matsui, K., Ajiki, T., Igarashi, Y., Takahashi, M., Murakami, T., Hakamata, Y., Tabata, Y., and Kobayashi, E. (2005). Can a bone marrow cell contribute to organ regeneration? In vivo analysis using transgenic rats with reporter genes. *Transplant Proc* 37, 273-275.

Welm, B., Behbod, F., Goodell, M. A., and Rosen, J. M. (2003). Isolation and characterization of functional mammary gland stem cells. *Cell Prolif* 36 Suppl 1, 17-32.

Yang, L., DeBusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., Matrisian, L. M., Carbone, D. P., and Lin, P. C. (2004). Expansion of myeloid immune suppressor Gr⁺CD11b⁺ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 6, 409-421.

kConFab Biospecimens Subcommittee

kConFab number:47/03-04

Applicants: Izhak Haviv

Date: 15 May 2005

The application is not approved. The response was reviewed by all members of the committee and the view was that it did not address the concerns of the committee.

1) The lack of obvious relevance to familial cancer remains a major concern. If studies on sporadic breast cancers reveal Y-chromosome markers an application to conduct a pilot study on kConFab cases may be considered.

2) If point 1 can be addressed and a re-application is made, the application must include (i) basic hypotheses and questions arising from this and (ii) a plan for experimentation and analysis that is clearly designed to address the questions. It would also be helpful to include the research plan from the DoD application.

Haviv Izhak

From: Devereux Lisa
Sent: Tuesday, 5 July 2005 9:20 AM
To: Haviv Izhak
Subject: RE: Breast ca donors

Dear Izi,

As your project has received EC approval already, it will just be a matter of requesting access to the TB samples from the TRMC. We have made DNA from approximately half of the cases and will continue to produce the DNA so that it will be ready for you when the TRMC approves. You will need to write a memo to the TRMC stating your wish to access DNA from the TB breast cancer patients, the amount you need. Also, do you want access to sections as stated in your original application? It would be clearest if you submit your full application as seen by the committee in March including the changes requested by the committee before it was submitted to the EC. The next deadline for the TRMC will be 19th July for the meeting on July 26th. I haven't heard back from the Royal Melbourne yet but will let you know asap.

Cheers,

Lisa

Lisa Devereux

Tissue Bank Manager
Peter MacCallum Cancer Clinic
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A'Beckett St, Melbourne 8006
9656 1102
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